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Effect of lemon (Citrus lemon) pumace powder supplementation on growth performance, lipid peroxidation and protein oxidation biomarkers in some tissues of common carp *(Cyprinus carpio)*

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ABSTRACT

The aim of this study was to evaluate the antioxidant potential of lemon pumace powder (peel and pulp), as an inexpensive and valuable source of nutrient in diet of common carp. Fish (60 ± 5 g) were divided randomly into four groups of 30 each. Group 1 fish were fed with basic diet, serving as the control. Fish in group 2 and 3 and 4 were fed the basic diet supplemented with 1.5%, 3% and 5% lemon pumace powder, respectively. Results showed that growth performance including final weight, weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR) increased significantly as compared to control. Malondialdehyde (MDA) values of muscle increased significantly as compared to control in all treatment groups and the decreeing effect of lemon pumace powder on malondialdehyde (MDA) values of kidney and liver was only significant in group 4, when compared with the control group ($P<0.05$). Protein carbonyl contents were decreased significantly in kidney and liver in group 3 and 4 as compared to control group and Protein carbonyl of muscle decreased significantly as compared to control in all treatment groups. FRAP values of liver increased significantly only in group 3 as compare to control and FRAP values of kidney and muscle increased significantly only in group 4 as compared to control ($P<0.05$). These data suggest that supplementation of 5% lemon pumace powder to be more effective than lower levels in strengthening the antioxidant system against oxidative stress.

Keywords

Antioxidant, Common carp, Lipid peroxidation, Protein carbonyls

Abbreviations

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Introduction

Fish lipid varies from that in mammalian lipid. The major difference is that lipids in fish accounts for about 40% of long chain fatty acids (14–22 carbon atoms) which are highly unsaturated. Mammalian lipids would partly involve up to two double bonds in every fatty acid molecule while as for fish depot fats it has enormous fatty acids with five or six double bonds. In addition, fish oils has other PUFAs which are served as 'essential' including eicosapentaenoic (EPA, C20:5n3) and docosahexaenoic (DHA, C22:6n3) acids among many others. In facts, EFSA [1] indicated that a daily consumption of 250–500mg of EPA and DHA lowers mortality risk results from coronary heart disorder and sudden heart failure. This confirms the past results that EPA in blood is an intensely powerful antithrombotic factor [2]. Notwithstanding, long-chain fatty acids are as vital as their high sensitivity to degradation, including oxidation. It has been established [3, 4, 5] that food lipid oxidation, in particular of PUFA contained in fish, is rather associated to of off-flavor components formation, quality degradation while enormous storage situation situations, loss of nutritional value and even anti-nutritional molecules formation. Oxidants are found to be reactive oxygen species (ROS) which have the potential, both directly and indirectly, to degrade all biomolecules, as proteins, lipids, DNA, and carbohydrates. ROS such as the superoxide anion, hydrogen peroxide and the hydroxyl radical are produced in the process of normal metabolism via electrons leakage from the electron transport chain and at the same time using the functions of different oxidoreductase enzymes [6]. Insufficient dietary antioxidants have been followed by a decrease in antioxidative defense and increased susceptibility to oxidative stress in both mammals and fish [7, 8]. The antioxidative defense system potential of aqua-cultured fish has been reported very poor [9]. The antibacterial, antioxidant and anticancer effects found in citrus are due to its high content of phenolic compounds particularly limonene. Lemon (Citrus limon) is the third most important species of citrus in the world, behind orange and mandarin. Production of lemon and lime in 2012 in FAO countries was 11.2 million tones. Lemon contain of amount compound such as calcium, potassium, magnesium, phosphor and vitamins including A, E, C, B12, B6 and flavonoid as a antioxidative agent as well [10]. So that the major portion of vitamin C located in lemon peel and pulp that acts as nutricidal for treatment of obesity, diabetes, blood lipid levels, cardiovascular diseases and cancer [11]. Lemon peels exhibits antimicrobial activities which are rich in flavonoid glycosides, coumarins, β and ϒ-sitosterols, and unpredictable compounds [12]. The unstable compounds are mixes of monoterpenes (limonene), sesquiterpens and sesquiterpenoids, for instance, aldehydes (citral), ketones, acids, liquor (linolel) and esters [13]. Rarely some researches have evaluated effects of various nutritious statuses on oxidative status biomarkers in angle species. As common carp is one of the most economically vital cultured fish species, it is necessary to identify its dietary requirements. It is worthy to explore the effects of dietary lemon pumace powder (peel and pulp) supplementation on oxidation biomarkers levels of proteins and lipids in various tissues of common carp.

Results

Analyzed proximate composition of lemon pumace powder and diet formulation and proximate composition of the basal diet is shown in Table 2.

GC-MS analysis

GC-MS chromatograms of lemon essential oil is shown in table 3. The most predominant compound of lemon essential oil was the limonene.

Growth and feeding parameters

The growth performance of common carp fed diets supplemented with varying levels of lemon pumace powder is presented in Table 4. At the end of 30 days experimental periods the survival was 100% in all groups. Fish fed diet supplemented diet including 3% and 5% lemon pumace powder did improved ($P < 0.05$) growth performance including final weight, weight gain (WG), feed conversion ratio (FCR) and specific growth rate (SGR).

Table 1
Composition and proximate analysis of diets.

Ingredient	(g/kg)
Fishmeal	300
Soybean meal	160
Corn meal	240
Wheat flour	180
Rice bran	80
Fish oil	20
Soybean oil	20

Table 2
Composition and proximate analysis of lemon pumace powder

Lemon pumace powder analyzed	(%)
Crude protein	8.4
Moisture	5.9
Ash	6.7
Dry matter	94.1

Table 3
GC Analysis of lemon essential oil

component	Retention time(min)	Area sum,%
3-methylheptane	4.16	0.01
1,3-dioxolane	4.32	0.72
Cis-1-ethyl-3-methyl-cyclopentane	4.51	1.42
n-octane	4.75	8.26
P-cymenene	5.65	4.13
linalool	6.92	1.12
fenchol	8.19	1.29
α-pinene	8.48	0.61
camphene	8.96	0.38
decane	10.71	2.08
m- cymenene	11.58	2.65
D-limonene	11.77	28.86
isoborneol	12.20	1.52
ϒ-terpinene	12.82	2.18
Terpinene-4-ol	12.97	1.05
α- terpineol	13.90	15.65
β-linalool	14.28	2.22
Fenchyl alcohol	14.78	1.47
Endo-borneol	16.66	1.71
α- terpineol	17.57	12.72
α- bergamotene	25.74	2.41
β-bisabolene	27.48	3.59
β-bisabolene	27.97	3.97

Biochemical assays and analysis

The effects of dietary lemon pumace powder supplementation on the levels of oxidation biomarkers of proteins and lipids in some tissues of common carp are presented in Figs. 1-4. As shown in Fig. 1, the decreeing effect of lemon pumace powder on malondialdehyde (MDA) values of kidney and liver was only significant in group 4, when compared with the control group ($P<0.05$). Muscle malondialdehyde (MDA) values increased significantly in all treatments comparing to control (fig. 2). Protein carbonyl contents were decreased significantly in kidney and liver in group 3 and 4 as compared to control group and protein carbonyl of muscle decreased significantly as compared to control in all treatment groups (Figs. 3, 4). As shown in Fig. 5, Group 3 showed significant increase in liver FRAP values comparing to control. Meanwhile, only group 4 had major increase in kidney and muscle FRAP contents compared to control (Fig. 5, 6).

Discussion

Potentially Lipid oxidation is affected by increase in PUFA content which in turn may affect color, flavor contributing oxidative stability in weak storage condition [15]. But, lipid oxidation can be avoided using dietary antioxidants. It has been clearly proved that αTAc supplementation led to optimal oxidative stability [16, 17, 18]. However, scholars about natural antioxidant supplementation feasibility, are growing recently. Our results showed an antioxidative dose ef-

Table 4
Growth performance of common carp fed various levels of lemon pumace powder

Group	Initial weight	Final weight	WG (%)	SGR (%)	FCR	Survival (%)
Control	61.12 ± 0.68	65.57 ± 0.62	7.33 ± 1.07^{a}	14.82 ± 2.1a	8.7 ± 2.7^{a}	100^{a}
Treatment (1.5%)	60.21 ± 1.12	65.80 ± 1.04	9.41 ± 1.55^{a}	18.61 ± 2.89a	6.1 ± 1.4^{a,b}	100^{a}
Treatment (3%)	68.13 ± 1.38	81.11 ± 1.24	19.23 ± 1.64^{b}	43.26 ± 3.08b	2.3 ± 0.15^{c}	100^{a}
Treatment (5%)	63.18 ± 1.15	85.27 ± 0.91	35.22 ± 1.9^{c}	73.64 ± 2.95c	1.2 ± 0.88^{c}	100^{a}

Values are mean ± SEM of each experimental group. Mean values with different superscripts are significantly different from each other (significance level is defined as $P < 0.05$).

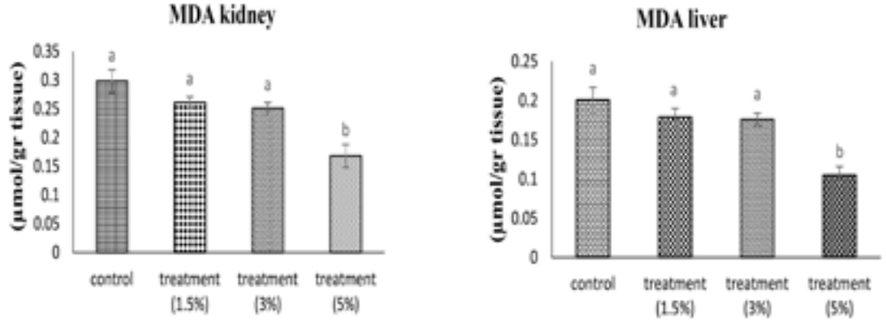

Figure 1.
Effect of dietary lemon pumace powder on malondialdehyde concentration in liver and kidney of common carp. Data are mean ± SEM (n=10 in each group).

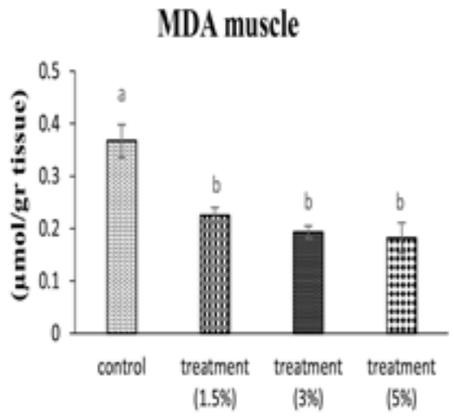

Figure 2.
Effect of dietary lemon pumace powder on malondialdehyde concentration in muscle of common carp. Data are mean ± SEM (n = 10 in each group).

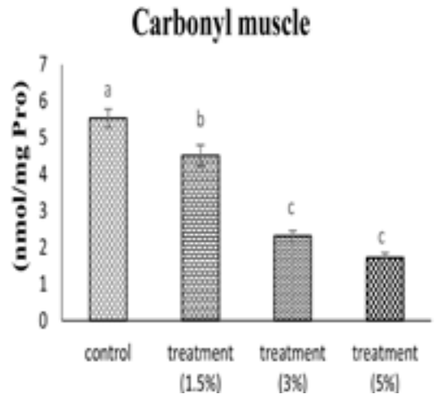

Figure 3.
Effect of dietary lemon pumace powder on protein carbonyl content in muscle and liver of common carp. Data are mean ± SEM (n = 10 in each group).

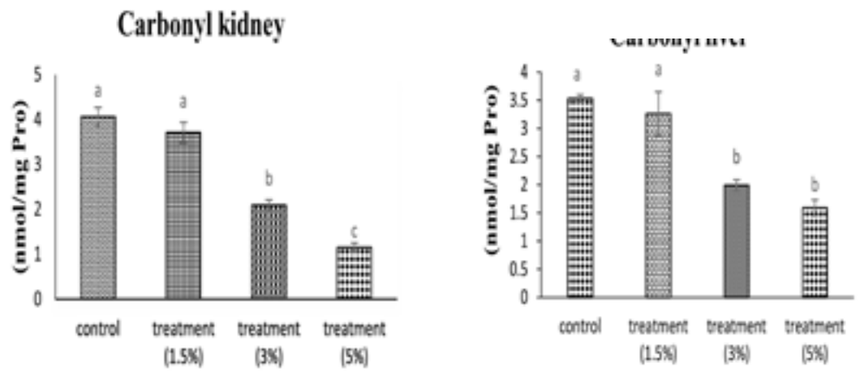

Figure 4.
Effect of dietary lemon pumace powder on protein carbonyl content in kidney and liver of common carp. Data are mean ± SEM (n = 10 in each group).

fect for natural antioxidant of lemon. These findings confirm the work of Lau et al [19] for grape seed and of Coetzee and Hoffman [17] for αTC. As for green tea, a pro-oxidative dose effect was found seen, which is in contradicted with those of Tang [20], who found a clear antioxidative dose-response effect. At the same time other plant extracts, rosemary and sage extracts at 500 ppm [18], oregano and rosemary essential oils at 150 and 300 ppm [15] or at 100 and 200 ppm [16, 21], and a combination of marigold, purple coneflower, black currant and yellow bark at 1000 ppm [16] have been shown to improve the oxidative stability of chicken meat. The antioxidant potential was also higher for lemon pumace, radical savaging activity of pumace in the DPPH assay was 82 ± 1.23 µg/ with IC50 83.061 µg/mL and the FRAP assay was 92.25 ± 2.45 µM trolox equivalents/g dry weight. The antioxidant properties and high content of flavonoids in the peels can make this waste material a good source of nutraceutical and healthy phenolic compounds, especially to be used as anti-ageing products, due to the high content of polyphenols. Previous researches have proved that plant extracts improve the immune system of fish using enhancing the innate and adaptive immune response against pathogens [22, 23]. Such extracts trigger both humoral and cellular defense mechanisms [24]. Plant extracts can enhance fish health status and immune potential, stimulating and leucopoiesis [25]. Many properties associated to an established digestive functions in mammals have been proved using citrus herbs, such as lemon, including increased appetite or prebiotic impacts [11]. In the current study, at the end of 30 days experimental period fish that were fed diet supplemented with 3% and 5% of lemon pumace powder displayed significantly established growth function including final weight, WG, FCR and SGR. This result is in line with those obtained in for O.mossambicus fed with citrus sinensis peel essential oil [26] and in Labeo victorianus fed with citrus lemon peel essential oil [27]. Nutrient supplementation in fish diets has been identified as a cost-effective approach for enhance function of different intensive fish production mechanisms [28]. Most of the researches including changing oxidative status biomarkers in aquatic animals have focused on stress resulted using salinity variations, temperature fluctuations, hypoxia, etc. Just a few have evaluated the impact of varying nutritional status upon oxidative status biomarkers in fish. Most cellular structure and function components are probably to be potential targets of oxidative degrade. Some analytical methods have been extended to assess the oxidation products directly (carbonyl assay for oxidized proteins) or the resultant degradation products (malondialdehyde for lipid peroxidation) [29]. Such oxidation products may be applied as biomarkers in tissue or plasma to assess the irreversible impacts of oxidative stress in animal [30, 31] and human's models [32, 33]. In addition, according to present result, dietary lemon pumace supplementation at 5% diet led to significant decrease in TBARS values in liver, kidney and muscle compared with the control group. In line with the present results, several researches have indicated decreasing impacts of garlic in lipid peroxidation in humans [34, 35, 36] and animal models [37, 38, 39, 40]. Kumar et al [41] at the same time have illustrated an ameliorative effect of garlic on lipid peroxidation in freshwater catfish Clarias batrachus while exposing to cadmium. In addition, some researches have indicated an enzymatic antioxidant system enhancement after garlic administration that could offer some protection against free-radical degradation [42, 43, 44]. As per the current results, protein carbonyl contents were lowered significantly in muscle, kidney and liver among others. According to present research results, cured garlic administration avoided the gentamicin-resulted rise in renal levels of protein carbonyl groups [45]. It has been reported that protein oxidation is associated to lipid oxidation in turkey meat [46] and fish fillet [47]. In fish, Srinivasan and Hultin [47] found a relationship between carbonyl content and TBARS values in cod when exposed to a free-radical-generating system. However, our data do not support such a possible relationship. In line with our findings, the study of Mercier et al [46] on beef showed an effect of diet on lipid oxidation but no significant dietary effect on protein oxidation. On the other hand, another study on lamb showed a significant effect of diet on meat protein oxidation that was not associated with a dietary effect on lipid oxidation [48]. But, it must be served that carbonyl production is just a general index of protein oxidation and other groups can be oxidized which do not forming carbonyls [47]. In the present research, a significant rise in FRAP value as a measure of total antioxidant status in muscle, kidney and liver tissue was found. It has been indicated that using some medicinal herbs and natural antioxidants in raising the total antioxidant status in different phases of meat preservation is efficient [49]. According to Norhaizan [50] it was found that some rice bran compounds can elevates the amount of FRAP value in some cell cultures significantly compared to the control group. As a whole this research indicated that using lemon pumace powders can enhance the oxidative status using lowering the oxidation of lipids and proteins in the muscle of common carp. Its application as a diet supplement in diet of fish can be applied to enhance the health status. In the past decades, plant-derived compounds have received a great deal of attentions mainly for their contributions in food preservation, particularly for the

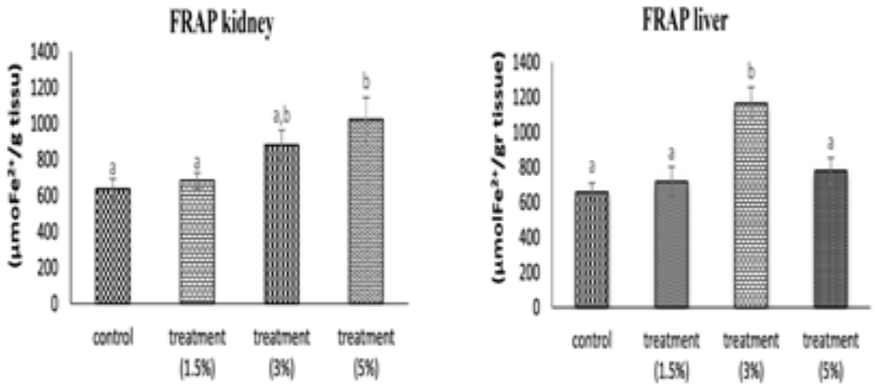

Figure 5.
Effect of dietary lemon pumace powder supplementation on frap concentration in kidney and liver of common carp. Data are mean ± SEM (n = 10 in each group).

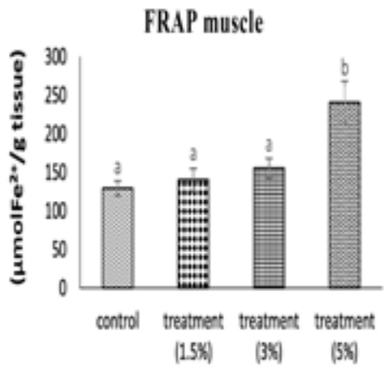

Figure 6
Effect of dietary lemon pumace powder supplementation on frap concentration in liver of common carp. Data are mean ± SEM (n = 10 in each group)..

stopping lipid oxidation. As a natural product, lemon pumace with high antioxidant potential have been increasingly preferred over synthetic antioxidants due to nontoxic nature and posing no health concerns. The results of the present study indicate that dietary lemon pumace powder supplementation can decrease oxidative stress to some extent using of antioxidant system improvement and lowering levels of lipid peroxidation and protein oxidation in some tissues of common carp. In addition, as per biochemical analysis results, it could be recommended that the lemon doses used in this research might have no damaging impacts on organs of common carp.

Materials and methods

Experimental design and sampling

A commercial pellet diet was crushed and mixed with tap water before adding the correct amount of crushed lemon pumace powder and pelleting to obtain diets supplemented with 0% (control), 1.5, 3 and 5 percent lemon pumace powder. Diet formulation and proximate composition of the basal diet were shown in Table 1.

One hundred and twenty common carp (Cyprinus carpio), weighing 60 ± 5 g, were obtained from a local farm (Mazandaran, Iran). They were divided randomly into 4 equal groups and held in four glass aquaria, each containing 250 L fresh water. Fish were acclimatized for 7 days before commencement of the experiment and were fed with a commercial pellet diet at a rate of 2% body weight day-1. Physicochemical conditions of the water during the experimental period were dissolved oxygen 5.5–6 ppm, temperature 25 ± 1 °C, pH 7 ± 0.5. Photoperiod was a 12:12 light– dark cycle the water in the aquaria was renewed every 48 h. Group 1 fish were fed with basic diet, serving as the control. Fish in groups 2, 3 and 4 were fed the basic diet supplemented with 1.5, 3 and 5% lemon pumace powder, respectively. The fish in each group were fed three times daily at 8:00, 13:00 and 19:00 throughout the experiment period (30 days). At the end of the experiment, 10 fish were selected randomly from each aquarium and anesthetized in diluted MS-222. Blood samples were taken by cardiac puncture using heparinized syringes and tubes. After plasma separation by centrifugation at 1000×g for 20 min, erythrocyte pellet was washed three times with normal saline solution. The washed centrifuged erythrocytes were hemolyzed by the addition of an equal volume of ice-cold redistilled water and prepared plasma hemolysate aliquots were stored at -70°C until analysis.

Preparation of lemon pumace powder

Lemon pumace (C. lemon) were obtain from a local lemon juice factory in Mashhad, Iran then the lemons (Citrus lemon) peel was dried under the shade in room temperature. After that, the dried peels were powdered using a mortar as well as electric blender.

Chemical analysis

Analyzed proximate composition of lemon pumace powder were determined according to the Method of AOAC (2002). Crude protein content was determined by Kjeldahl method using an Auto Kjeldahl System (Kjeltec™ 2300, Foss, Sweden). Moisture content by a dry Measurement of protein percentage, ash percentage, moisture content, calcium, phosphorus and dry matter content were measured by AOAC (2002) method.

GC-MS analysis (ISO 7609:1985)

GC-MS analysis was performed on Agilent Technology (Little Falls, California, USA) 6890 series gas chromatography (GC) system, equipped with 5973 mass spectrometry (MS) detector and a 7683 series auto-injector was used. Compounds were separated on Rtx®-Wax capillary column (30 m × 0.25 mm, film thickness 0.25 µm; RESTEK, Pennsylvania, USA). Helium (5N5 grade) was used as carrier gas, with a flow rate of 0.8 mL/min, and the split ratio was 60:1. Sample injection volume was 1 µl and the injector temperature was 230°C. The column oven temperature was held at 70°C for 2 min, and then programmed to 130°C at 30°C/min and change the gradient to 230°C with 10°C/ min. Finally, held at 230°C for 6 min and the total run time was 20 min. An electron ionization (EI) system with ionization energy 70 eV was used for detection. The ion source temperature was set at 230°C, the interface temperature was 250°C, detector voltage was 2 kV. The mass spectrum was acquired in scan mode at a scan rate 0.98 scan/sec within a mass range of 20-800 amu. The measurement was performed in duplicate for each sample with solvent delay for 2 min.

Antioxidant Assessment

Free Radical Scavenging Activity: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals absorb at 517 nm, but upon reduction by an antioxidant compound, absorption decreases. Briefly, 50 µL of processed SPE MeOH extract or pure compound prepared at different concentrations was added to 2 mL of fresh 0.1 Mm solution of DPPH in methanol and allowed to react at 37 °C in the dark. After thirty minutes the absorbance was measured at 517 nm [51]. The DPPH scavenging ability as percentage was calculated as: DPPH scavenging ability = (Acontrol – Asample/Acontrol) × 100.

Ferric Reducing Antioxidant Power

The determination of ferric reducing antioxidant power or ferric reducing ability (FRAP assay) of the extracts was performed as described by Benzie [14] with some modifications. The stock solutions prepared were 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) solution in 40 mM HCl, and 20 mM FeCl3·6H2O solution. Plant extracts or standard methanolic Trolox solutions (150 µL) were incubated at 37 °C with 2 mL of the FRAP solution (prepared by mixing 25 mL acetate buffer, 5 mL TPTZ solution, and 10 mL FeCl3·6H2O solution) for 30 min in the dark. Absorbance of the blue ferrous tripyridyltriazine complex formed was then read at 593 nm.

Chemicals

2,4-Dinitrophenylhydrazine (DNPH) and 2-thiobarbituricacid (TBA) were purchased from Sigma Chemical Co. (St.Louis, MO, USA). The rest of the utilized chemicals were of analytical grade and were supplied by Sigma (St. Lewis, MO, USA) or Merck (Darmstadt, Germany).

Biochemical assays and analysis

Tissue samples including muscle, kidney and liver were rapidly thawed and homogenized in 10 volumes (w/v) of ice-cold 0.05 M phosphate buffer (pH 7.4) for 5 min, and centrifuged at 4,000g for 15 min at 4 °C; the supernatant was kept in ice until assayed. Determination of malondialdehyde (MDA) concentration was based on spectrophotometry of the pink-colored product of thiobarbituric acid reactive substances, as described by Latha and Pari. The concentration of MDA was calculated using a molar extinction coefficient value of 156,000 M-1 cm-1. Carbonyl groups of proteins were detected by reaction with 2, 4-dinitrophenylhydrazine, which leads to the formation of a stable 2, 4-dinitrophenylhydrazone product (Petron et al., 2007). Resulting 2, 4 dinitrophenylhydrazones were quantified spectrophotometrically at 370 nm using a molar extinction coefficient of 22,000 M-1 cm-1.

Growth performance

All fish in the different experimental groups were weighed at the end of 30 days feeding trial for estimation of growth. Growth performance parameters were calculated according to the following formulae:
Weight Gain (WG) = (final weight-initial weight) × 100 / (initial weight)
Specific Growth Rate (SGR) = (final weight - initial weight) × 100 / days.
Feed Conversion Ratio (FCR) = feed given (dry weight) / total wet weight gain.
Survival= 100 × (final fish number / initial fish number).

Statistical analysis

The data (means ± SD) were analyzed by using one way analysis of variance (ANOVA) followed by Duncan's post hoc test to compare the means between treatments and differences were considered significant when P<0.05.

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Author Contributions

Conceived and designed the experiments: A.S., D.S., H.B. Performed the experiments: S.S., A. S., D.S., H.B. Analyzed the data: S.S. Contributed reagents/ma¬terials/analysis tools: H.B., S.S. Wrote the paper: S.S.

Conflict of Interest

None.

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